STIC-ILL

From: Schmidt, Mary
Sent: Monday, October 28, 2002 3:51 PM
To: STIC-ILL
Subject: references 09/909,796

Hi, please locate the following references:

Ploszaj et al., Amino acids (Austria), 2000, 19 (2), p483-96.

Stefanelli et al. Biochemical journal (England) May 1, 2000, 347 Pt. 3, p875-80.

Sakagami et al. Anticancer Research (Greece), Jan-Feb. 2000, 20 (1A), p265-70.

Ray et al., American journal of physiology, Cell physiology (US), Mar. 2000, 278 (3), pC480-9.

Bock et al. Radiation research (US), Dec. 1999, 152 (6), p604-10.

Dai et al. Cancer research (US), Oct. 1, 1999, 59 (19), p4944-54.

Bratton et al. Jo. of biological chemistry (US), Oct. 1, 1999, 274 (40), p28113-20.

Palyi et al. Anti-cancer drugs (England), Jan 1999, 10 (10, p103-11.

Li et al. Am. journal of physiology , April 1999, 276 (4 Pt. 1), pC946-54.

10 Ray et al. Am. journal of physiology, Mar. 1999, 276 (3 Pt. 1) pC684-91.

Das et al. Oncology Research (US), 1997, 9 (11-12), p565-72.

Monti et al.. Life Sciences (England), 1998, 62 (9), p799-806.

Lin et al., Experimental cell research, (US), Nov. 25, 1997.

Tome et al. biochemical Journal (England) Dec. 15, 1997, 328 (Pt. 3), p847-54.

Hu et al., Biochemical journal (England), Nov. 15, 1997, 328 (Pt. 1), p307-16.

Tome et al. biological signals (Switzerland), May -Jun 1997, 6 (3), p150-6.

Taguchi et al., Cell biochemistry and function (England), Mar 2001, 19 (1), p19-26.

Camon et al. neurotoxicology (US), Fall 1994, 15 (3), p759-63.

Shinki et al., Gastroenterology (US), Jan 1991, 100 (1), p113-22.

Heston et al. Prostrate (US), 1982, 3 (4), p383-9

Stefanelli et al, biochemical journal (England), Apr. 1, 2001. 355 (pt. 1), p199-206.

Lopez et al., biocell: official journal of the sociedades latinoamericanas de microscopia electronica... et. al. 9Argentina), Dec. 1999, 23 (3), p223-8.

Schipper et al. seminars in cancer biology (US), feb. 2000, 10 (1), p55-68.

Nilsson et al., biochemical journal (England) Mar. 15, 2000, 346 Pt. 3, p699-704.

giuseppina monti m. et al., biochemical and biophysical research commun. (US), Apr. 13, 1999, 257 (2), p460-5.

ratasirayakorn et al, j. of periodontology feb. 1999, 70 (2), p179-84

stabellini et al., Experimental and molecular parhology (US), 1997, 64 (3), p147-55.

Sparapani et al, experimental neurology (US), nov. 1997, 148 (1), p157-66..

Dhalluin et al., carcinogenesis (Eng.), Nov. 1997, 18 (11), p2217-23.

Polyamine depletion arrests cell cycle and induces inhibitors $p21^{Waf1/Cip1}$, $p27^{Kip1}$, and p53 in IEC-6 cells

RAMESH M. RAY,[1] BARBARA J. ZIMMERMAN,[1] SHIRLEY A. McCORMACK,[1]
TARUN B. PATEL,[2] AND LEONARD R. JOHNSON[1]
Departments of [1]Physiology and Biophysics and [2]Pharmacology, College of Medicine, University of Tennessee, Memphis, Tennessee 38163

Ray, Ramesh M., Barbara J. Zimmerman, Shirley A. McCormack, Tarun B. Patel, and Leonard R. Johnson. Polyamine depletion arrests cell cycle and induces inhibitors $p21^{Waf1/Cip1}$, $p27^{Kip1}$, and p53 in IEC-6 cells. *Am. J. Physiol.* 276 (*Cell Physiol.* 45): C684–C691, 1999.—The polyamines spermidine and spermine and their precursor putrescine are intimately involved in and are required for cell growth and proliferation. This study examines the mechanism by which polyamines modulate cell growth, cell cycle progression, and signal transduction cascades. IEC-6 cells were grown in the presence or absence of DL-α-difluoromethylornithine (DFMO), a specific inhibitor of ornithine decarboxylase, which is the first rate-limiting enzyme for polyamine synthesis. Depletion of polyamines inhibited growth and arrested cells in the G_1 phase of the cell cycle. Cell cycle arrest was accompanied by an increase in the level of p53 protein and other cell cycle inhibitors, including $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. Induction of cell cycle inhibitors and p53 did not induce apoptosis in IEC-6 cells, unlike many other cell lines. Although polyamine depletion decreased the expression of extracellular signal-regulated kinase (ERK)-2 protein, a sustained increase in ERK-2 isoform activity was observed. The ERK-1 protein level did not change, but ERK-1 activity was increased in polyamine-depleted cells. In addition, polyamine depletion induced the stress-activated protein kinase/c-Jun NH_2-terminal kinase (JNK) type of mitogen-activated protein kinase (MAPK). Activation of JNK-1 was the earliest event; within 5 h after DFMO treatment, JNK activity was increased by 150%. The above results indicate that polyamine depletion causes cell cycle arrest and upregulates cell cycle inhibitors and suggest that MAPK and JNK may be involved in the regulation of the activity of these molecules.

ornithine decarboxylase; DL-α-difluoromethylornithine; putrescine; signal transduction; mitogen-activated protein kinase; cyclin-dependent kinase inhibitor

THE POLYAMINES SPERMIDINE and spermine and their precursor putrescine are intimately involved in and required for cell growth and proliferation (43, 57). Intracellular polyamine levels are highly regulated and are primarily dependent on the activity of ornithine decarboxylase (ODC), which catalyzes the first rate-limiting step in polyamine biosynthesis (48, 37). An increase in ODC activity is one of the earliest biochemical events associated with the induction of cellular proliferation (43, 57), and depletion of polyamines by DL-α-difluoromethylornithine (DFMO), a specific and irreversible inhibitor of ODC, attenuates trophic responses in a number of tissues (43, 37). Although the importance of polyamines in cell growth is well known, their exact role in specific events related to cell proliferation at the molecular level is still unclear.

Growth regulatory consequences of polyamine depletion suggest a link between signal transduction cascades, cell cycle machinery, and apoptosis. Several investigators have provided evidence for polyamine-dependent restriction points during the G_0-G_1 transition and G_1 phase in various cell types (5, 18, 51). So far, the precise location of the block has not been determined. Orderly progression through the cell cycle is now known to be dependent on the coordinated interaction between key cell cycle regulatory molecules including cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitory proteins such as $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ (20, 22, 25, 42). Increased levels of CDK inhibitory proteins prevent the release of E2F, a transcription factor required for the transcription of S phase genes, from retinoblastoma protein, resulting in cell cycle arrest (6, 7, 11, 33).

It is now established that the mitogen-activated protein kinase (MAPK) cascade is also involved in a variety of biological processes, such as differentiation (9, 58), cell attachment (8), cell motility (26), smooth muscle contraction (1), and protein synthesis (53), depending on the stimuli and cell type. For example, in PC-12 cells, epidermal growth factor (EGF) activation of the MAPK pathway leads to proliferation, whereas nerve growth factor-stimulated MAPK activation leads to differentiation (9, 58). Recently, a novel paradigm has been described in which the MAPK kinase (MEK)/MAPK pathway negatively regulates CDK activity and mediates cell cycle arrest (45).

The c-Jun NH_2-terminal kinase [JNK; also known as stress-activated protein kinase (SAPK)] group of MAPKs is activated by exposure of cells to environmental stress or by treatment of cells with proinflammatory cytokines (17, 21, 28, 56), ultraviolet light (10), DNA-damaging drugs (59, 64), protein synthesis inhibitors (21), ceramide (62), and tumor necrosis factor-α and interleukin-1 (28, 47). In addition, JNK activity is also induced by mitogenic signals, including growth factors (40) and oncogenic Ras (10). One potential function of JNK may be the initiation of programmed cell death (apoptosis). Overexpression of MEK kinase, the JNK kinase kinase, has a lethal effect on fibroblasts (23, 30). In addition, the tumor supressor p53, which is essential for radiation-induced apoptosis (40), may be a substrate of JNK-1 in vivo (39).

In the small intestine, cell proliferation and cell migration are fundamental processes for the organiza-

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

tion and maintenance of tissue integrity. Highly regulated mechanisms appear to control the rate of cell proliferation, migration, and differentiation of the crypt cells. Frey et al. (15) established that increases in $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ and hypophosphorylation of retinoblastoma protein are associated with phorbol ester-mediated activation of the protein kinase C isoform and cell cycle arrest in IEC-18 cells. Sustained elevation of JNK and p53 has been reported to induce apoptosis in several cell lines (8, 32). Spontaneous apoptosis is part of the stem cell homeostatic process, ensuring that extra stem cells are deleted from the small intestine. Addition of a single stem cell to the normal complement of 250 cells in a small intestinal crypt could distort the architecture of the crypt and generate a hyperplastic crypt, which is rare in the small intestine, implying that stem cell number is tightly regulated. Our laboratory has been interested in the role of polyamines in the growth and repair of the gastrointestinal mucosa and has examined these processes in both rats and in cultured, normal rat intestinal epithelial cells (IEC-6). Recently, we demonstrated that, in IEC-6 cells, EGF treatment decreased the doubling time by 8 h, whereas, in polyamine-depleted cells, EGF had no effect on the doubling time. An inhibitory effect of polyamine depletion on phosphorylation of the EGF receptor suggested the involvement of polyamines in signaling cascades (35). However, the precise role of polyamines in the signaling cascade that includes MAPK, JNK, and cell cycle regulators has not been investigated. In this report, data are presented that show the induction of p53 and CDK inhibitory proteins upon polyamine depletion and cell cycle arrest. Concomitant induction of MAPK and JNK during polyamine depletion suggests a link between the signal transduction cascade and negative growth regulation in IEC-6 cells.

MATERIALS AND METHODS

Materials. Medium and other cell culture reagents were obtained from GIBCO BRL (Grand Island, NY). Fetal bovine serum (FBS), dialyzed fetal bovine serum (dFBS; 1,000-molecular weight cutoff), myelin basic protein (MBP), and propidium iodide were from Sigma (St. Louis, MO). [γ-^{32}P]ATP and an enhanced chemiluminescence Western blot detection system were purchased from DuPont-NEN (Boston, MA). DFMO was a gift from the Merrell Dow Research Institute of Marion Merrell Dow (Cincinnati, OH). Both anti-MAPK extracellular signal-regulated kinase (ERK)-2 (monoclonal) and anti-MAPK ERK-1-CT (polyclonal) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). JNK-1, p21, p27, and p53 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The apoptosis detection kit was purchased from Oncor (Gaithersburg, MD). TACS apoptotic DNA laddering kit was purchased from Trevigen (Gaithersburg, MD). The IEC-6 cell line (American Type Culture Collection CRL-1592) was obtained from the American Type Culture Collection (Manassas, VA) at *passage 13*. The cell line was derived from normal rat intestine and was developed and characterized by Quaroni et al. (46). The cells are nontumorigenic and retain the undifferentiated character of epithelial stem cells. All other chemicals were of the highest purity commercially available.

Cell culture. The IEC-6 cell stock was maintained in T-150 flasks in a humidified, 37°C incubator in an atmosphere of 90:10 air-CO_2. The medium consisted of DMEM with 5% heat-inactivated FBS and 10 μg/ml insulin and 50 μg/ml gentamicin sulfate. The stock was passaged weekly at 1:10 and fed three times per week, and *passages 15–20* were used. For the experiments, the cells were taken up with 0.05% trypsin plus 0.53 mM EDTA in Hanks' balanced salt solution without calcium and magnesium and counted by hemocytometer.

Growth studies. The cells were plated at a density of 6.25×10^4 cells/cm^2 in T-25 flasks in DMEM containing 5% dFBS plus 10 μg/ml insulin and 50 μg/ml gentamicin sulfate (DMEM-dFBS) with or without DFMO or putrescine. Each experiment contained three each of untreated, DFMO-treated, and DFMO/putrescine-treated flasks. Every other day, the cells in three flasks from each group were taken up with trypsin-EDTA and the number of cells in an aliquot was counted by Coulter counter. Each experiment was carried out in triplicate, the controls were combined, and the data were expressed as percentages of control.

Apoptosis. Cells were plated as described above in DMEM-dFBS with or without DFMO or putrescine on chambered slides. On *day 5*, the cells were fixed in 4% neutral buffered Formalin and postfixed in 2:1 ethanol-acetic acid. Apoptotic cells were identified by terminal deoxynucleotide transferase labeling according to the manufacturer's instructions. Following the procedure, the cellular DNA was counterstained with propidium iodide (5 μg/ml). The nucleosomal fragmentation assay was carried out by isolating DNA from the cells using the TACS apoptotic DNA laddering kit and analyzing the DNA by agarose gel electrophoresis following the manufacturer's instructions.

Cell cycle analysis. Cells were plated in DMEM-dFBS with or without DFMO in T-25 flasks at a density of 6×10^4 cells/cm^2. They were taken up with trypsin-EDTA on *days 1–4* postplating. The cells were collected by centrifugation for 5 min at 100 *g*, washed with ice-cold PBS-1% BSA (washing buffer), resuspended in 0.5 ml, and fixed with 1 ml 70% ethanol at −20°C, added dropwise. The cells were washed three times in washing buffer at 4°C and resuspended in 1 ml containing 100 μg RNase (RASE, Worthington) and 5 μg propidium iodide. They were then incubated at 37°C for 15 min in the dark, washed three times with 3 ml of washing buffer, resuspended in 1 ml, and analyzed by flow cytometry.

Preparation of cell extract. The IEC-6 cells were plated (*day 0*) in 60-mm dishes at a density of 6.25×10^4 cells/cm^2 in DMEM-dFBS with or without DFMO (5 mM) and putrescine (10 μM). Dosages were chosen from dose-response curves carried out previously. On *day 3*, the medium was removed and medium was added that did not contain dFBS but did contain DFMO or DFMO plus putrescine when required. On *day 4*, medium was removed and 500 μl of cold immunoprecipitation buffer (IPB; 10 mM Tris·HCl at pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 200 μM Na_3VO_4, 80 μg/ml leupeptin, and 40 μg/ml aprotinin) was added. The cell suspension was rotated at 4°C for 30 min, and the extract was cleared by centrifugation at 10,000 *g* for 5 min. The extracts were stored at −80°C until use. Protein concentration was determined by the method of Bradford (4), using BSA as a standard.

Immunoprecipitation. Cell lysates were matched for protein and precleared with 20 μl of protein A/G agarose for 1 h at 4°C. The precleared supernatants were further incubated overnight with 2 μg of antibody recognizing p21, p27, p53, ERK-1, ERK-2, or JNK-1, and immunocomplexes were pread-

sorbed to protein A/G agarose. Preadsorbed immunocomplexes were then used to measure ERK-1, ERK-2, or JNK activity by in vitro kinase assay and p21, p27, and p53 by Western blotting.

In vitro MAPK assay. The immunocomplexes were pelleted by centrifugation (10,000 *g* for 2 min) and then washed three times with IPB and once with kinase assay buffer [KAB; 20 mM Tris·HCl, pH 7.4, 10 mM $MgCl_2 \cdot 6H_2O$, 200 μM Na_3VO_4, 10 mM NaF, and 1 mM dithiothreitol (DTT)]. The pellet was resuspended in 40 μl KAB containing 0.3 mg/ml MBP, 20 μM ATP, and 10 μCi [γ-^{32}P]ATP. After 20 min at 30°C, the reaction was terminated by the addition of 40 μl of 2× Laemmli sample buffer. Samples were then boiled for 5 min and subjected to 15% SDS-PAGE. The gels were stained with Coomassie brilliant blue, dried, and exposed for 1–3 h to Kodak X-ray film.

In vitro JNK assay. The immunocomplexes were pelleted by centrifugation (10,000 *g* for 2 min) and then washed three times with JNK buffer and once with JNK assay buffer. The agarose beads were resuspended in 30 μl JNK assay buffer (20 mM HEPES, pH 7.6, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 2 mM DTT, 100 μM *p*-nitrophenyl phosphate, 100 μM Na_3VO_4), 1 μg of glutathione *S*-transferase (GST)-c-Jun protein, 20 μM ATP, and 10 μM [γ-^{32}P]ATP. After 20 min at 30°C, the reaction was terminated by the addition of 30 μl of 2× Laemmli sample buffer. Samples were then boiled for 5 min and subjected to 15% SDS-PAGE. The gels were stained with Coomassie brilliant blue, dried, and exposed for 1–3 h to Kodak X-ray film.

Western blot analysis. Cell extracts were prepared as described above. Total cell protein (50 μg) was separated on 15% SDS-PAGE gels and transferred to nitrocellulose membranes for Western blotting. Equal loading of protein was confirmed by staining the nitrocellulose membrane with Ponceau S. The membranes were then probed with an antibody directed against one of the proteins (p21, p27, p53, ERK-1, ERK-2, and JNK-1). The immunocomplexes were visualized by the enhanced chemiluminescence detection system and quantitated by densitometric scanning.

RESULTS

Polyamine depletion inhibits growth but does not induce apoptosis in IEC-6 cells. Several investigators have demonstrated that polyamines are required for cell growth and proliferation (43, 48, 57). In this and other studies (35), we have shown that depletion of cellular polyamines by long-term administration of DFMO significantly decreases IEC-6 cell growth. Decreased cell growth occurred within 3–4 days following DFMO treatment and remained unchanged (Fig. 1). Supplying exogenous spermidine prevented the decrease in rate of growth. Similar restoration of growth occurs with putrescine or spermine. Recently, studies have shown that increases in JNK result in the induction of apoptosis in certain cell types (8). We wanted to examine whether the reduction in growth in polyamine-depleted cells was a result of apoptosis. By examining the terminal deoxynucleotide transferase labeling of 3′-OH ends of DNA (Fig. 2*A*) and DNA laddering (Fig. 2*B*) as markers of apoptosis, we found that treatment of IEC-6 cells with DFMO did not result in the induction of cell death.

Polyamine depletion affects the progression of the IEC-6 cell cycle. The increased doubling time and

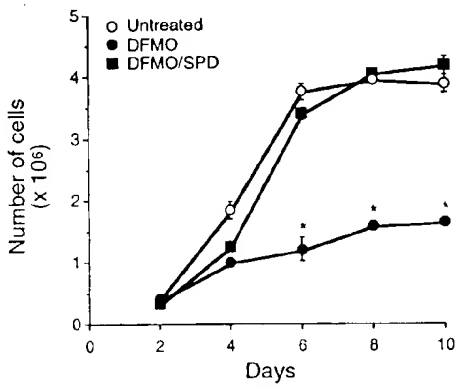

Fig. 1. Polyamine depletion decreases cell growth. Growth curves of IEC-6 cells that were grown under control conditions or in presence of 5 mM DL-α-difluoromethylornithine (DFMO) or DFMO + 5 μM spermidine (SPD) (means ± SE, n = 3). *Significant difference compared with untreated controls using ANOVA ($P < 0.05$).

decreased cell number without apoptosis suggest that polyamines might be involved in the regulation of the cell cycle. Therefore, we examined the effect of DFMO on the progression of the cell cycle. In controls, normal cell cycle progression was evident by an increased S phase with a concomitant decrease in the G_1 phase on *days 1* and *2* and then subsequent accumulation of cells into G_1 on *day 3* when cells reached confluence (Fig. 3*A*). In contrast to this, polyamine depletion arrested cells in the G_1 phase on *day 1*, as shown in Fig. 3*B*. About 60% of the cells were in the G_1 phase, and this increased on subsequent days. Addition of putrescine to DFMO-treated cultures caused the normal progression of the cell cycle to resume (data not shown).

Polyamine depletion induces p53, p21$^{Waf1/Cip1}$, and p27^{Kip1}. As shown in Fig. 4, DFMO treatment of IEC-6 cells results in the accumulation of proteins that inhibit cell cycle progression. The level of p53, a tumor supressor gene product, was increased by 150% in DFMO-treated cells (Fig. 4*A*); p27^{Kip1}, a mitotic inhibitor protein, was upregulated in polyamine-depleted cells by 50% (Fig. 4*B*). Polyamine depletion also resulted in a remarkable increase (77%) in the level of p21$^{Waf1/Cip1}$ (Fig. 4*C*), which negatively regulates the formation of cyclin-CDK complexes that are required for normal cell cycle progression. These results support the notion that polyamines normally suppress pathways that increase CDK inhibitory protein expression.

Effect of polyamine depletion on the activities and levels of ERKs. We previously reported that polyamines are required for EGF-mediated cell proliferation in IEC-6 cells (35). It is now well established that the EGF-mediated signal cascade that involves ERKs (ERK-1, ERK-2, and JNK, also called SAPK) plays important roles in cell proliferation (50). Phosphorylation of transcription factors such as c-Fos, c-Myc, and c-Jun by activated ERKs increases the rate of transcription of growth-related genes. Previous studies from this laboratory have established that DFMO treatment depleted putrescine within 3 h, but depletion of spermidine required 24 h and depletion of spermine required

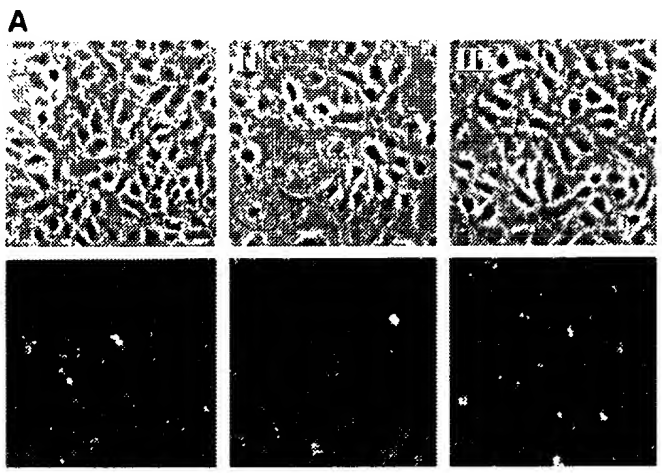

Fig. 2. Polyamine depletion does not induce apoptosis in IEC-6 cells. *A*: cells were grown for 4 days under control conditions (*I*) or in presence of DFMO (*II*) or DFMO + putrescine (*III*) and then were subjected to terminal deoxynucleotide transferase labeling. Immunofluorescence analysis of each slide is shown below corresponding phase-contrast photomicrograph. *B*: cells were grown as described above, and isolated DNA was subjected to agarose gel electrophoresis to determine nucleosomal cleavage by DNA laddering assay. Molecular weight (MW) lanes are DNA size markers λ*Hind* III digest (*left*) and ϕX174 *Hae* III digest (*right*).

3 days (36). We examined the activities of ERK-1 and ERK-2 by phosphorylation of MBP and JNK activity by phosphorylation of GST-c-Jun protein in DFMO-treated cells (3 days), which were then serum deprived to achieve quiescence. Polyamine depletion caused an ~50% increase in ERK-1 activity, and supplementation with putrescine restored the basal level of ERK-1 activity (Fig. 5, *A* and *B*). Polyamine depletion had only a marginal effect on the level of ERK-1 protein (Fig. 5*C*). In contrast, polyamine depletion increased ERK-2 activity 150% compared with control (Fig. 6). The increase in ERK-2 activity was largely prevented by addition of putrescine to DFMO-treated cells (Fig. 6, *A* and *B*). Interestingly, polyamine depletion significantly reduced the level of ERK-2 protein, which was also prevented by putrescine (Fig. 6*C*). Similarly, the activity of JNK was elevated upon polyamine depletion (120%) and remained elevated in the presence of putrescine (Fig. 7, *A* and *B*). The level of JNK protein was low in polyamine-depleted cells and returned to normal with the addition of putrescine (Fig. 7*C*). The induction of JNK activity was the earliest response to polyamine depletion, increasing within 5 h of DFMO treatment (Fig. 8). This increase was prevented by putrescine.

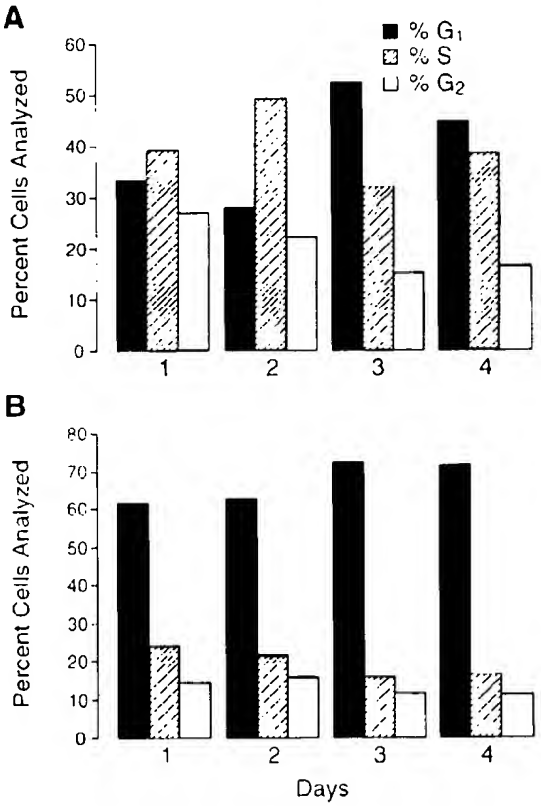

Fig. 3. Effect of polyamine depletion on cell cycle progression. IEC-6 cells were grown in medium without (*A*) and with (*B*) DFMO. At indicated time intervals, cells were processed as described in MATERIALS AND METHODS and subjected to flow cytometric analysis. Percentages of total cells analyzed that are in G_1, S, and G_2 phases are shown from a representative of 3 experiments.

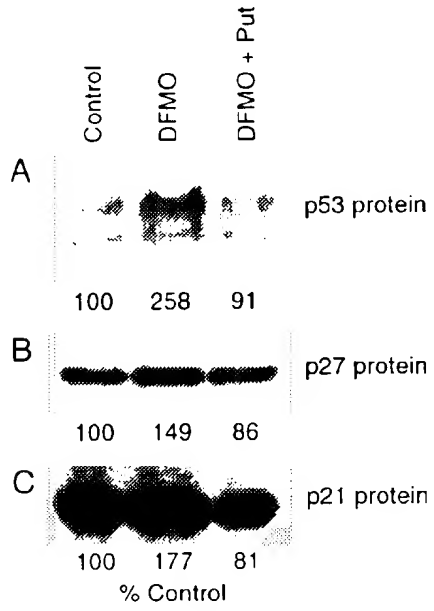

Fig. 4. Polyamine depletion increases levels of p53 (*A*), p27 (*B*), and p21 (*C*) proteins in IEC-6 cells as indicated by Western blot analysis.

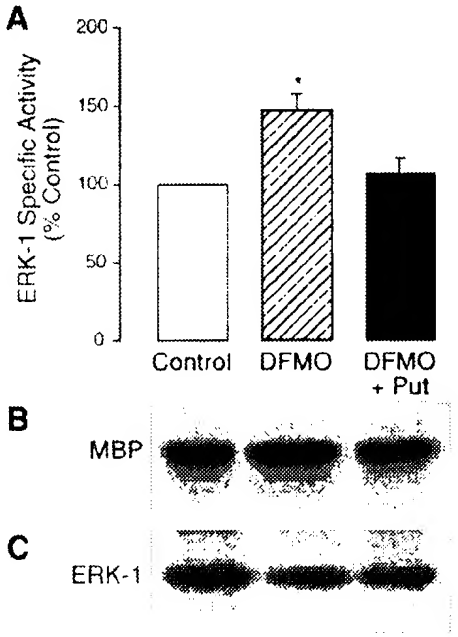

Fig. 5. Effect of polyamine depletion on activity and level of extracellular signal-regulated kinase (ERK) 1. IEC-6 cells were grown under control conditions or in presence of DFMO or DFMO + putrescine for 3 days and serum starved for 24 h, and then cell extracts were prepared. After immunoprecipitation, ERK 1 activity was measured by phosphorylation of myelin basic protein (MBP, *B*) and protein level (*C*) was analyzed by Western blot analysis. Specific activity (*A*) is expressed as %control (means + SD, $n = 3$) and was calculated from arbitrary units of activity and protein levels determined by densitometric scanning. *Significant difference compared with control using ANOVA ($P < 0.05$)

These data indicate that polyamines influence the activities and protein levels of signal-regulated proteins in IEC-6 cells. These observations suggest that increases in the activities of ERKs as a result of polyamine depletion may be related to the inhibition of cell proliferation.

DISCUSSION

The rapidly proliferating cells of the mucosal epithelium of the small and large intestines constitute a dynamic and unique system to study factors regulating cell growth and differentiation. Increased understanding of the molecular factors that promote growth and maturation of the intestinal epithelium has considerable therapeutic relevance. The gastrointestinal mucosa has the ability to repair itself rapidly after damage. Repair of the damaged mucosa, called mucosal restitution or reepithelialization, consists of the sloughing of damaged epithelial cells, movement of surrounding cells to cover the wounded area (migration), and finally replacement of lost cells by the process of cell division (proliferation) (55). A series of studies from this laboratory established that polyamines are essential for the normal repair of gastric and duodenal erosions induced in a rat stress ulcer model. DFMO, an enzyme-activated inhibitor of ODC, almost completely prevents healing, and oral administration of polyamines immediately after the period of stress restores the normal rate of healing (60, 61). Although it has been repeatedly demonstrated that, in most cells, polyamine depletion leads to an inhibition of cell proliferation, the actual mechanism by which this is achieved is not understood at the molecular level. In view of the above, in the present investigation we demonstrate that polyamine depletion leads to cell cycle arrest with the induction of p53, p27, and p21 and involvement of sustained induction of the MAPK signal transduction cascade.

Because polyamine depletion had such a dramatic effect on cell growth (Fig. 1), we presumed that polyamines might have some regulatory role in cell cycle progression. IEC-6 cell cycle analysis was carried out in the presence and absence of DFMO to establish the specific effect of polyamine depletion on cell growth. Results in Fig. 3*B* clearly indicate that polyamine depletion arrested cells in the G_1 phase within 24 h (i.e., within one cell cycle). Seidenfeld et. al. (51) also showed a marked increase in the G_1 phase fraction and decrease in the S phase fraction as a consequence of DFMO treatment in four different carcinoma cell lines. More recently, Fredlund and Oredsson (13, 14) found impaired DNA replication and lengthening of the G_1

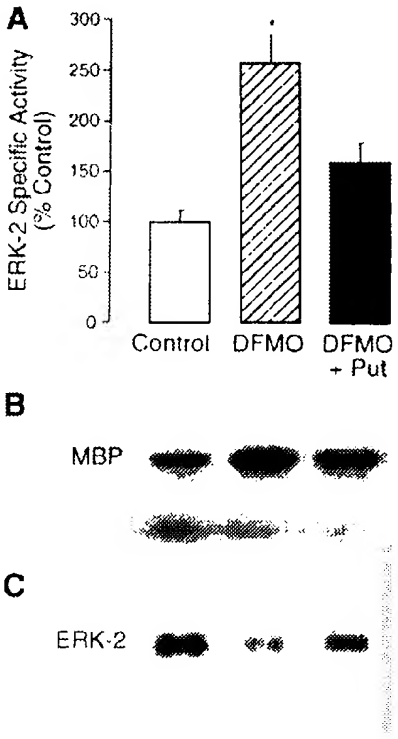

Fig. 6. Effect of polyamine depletion on activity and level of ERK-2. IEC-6 cells were grown under control conditions or in presence of DFMO or DFMO + putrescine for 3 days and serum starved for 24 h, and then cell extracts were prepared. After immunoprecipitation, ERK-2 activity was measured by phosphorylation of MBP (*B*) and protein level (*C*) was analyzed by Western blot analysis. Specific activity (*A*) is expressed as %control (means ± SD, $n = 3$) and was calculated from arbitrary units of activity and protein levels determined by densitometric scanning. *Significant difference compared with control using ANOVA ($P < 0.05$).

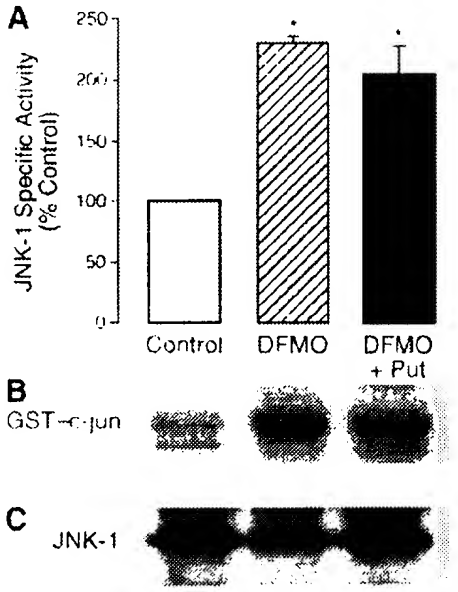

Fig. 7. Effect of polyamine depletion on activity and level of c-Jun NH_2-terminal kinase (JNK) 1. IEC 6 cells were grown under control conditions or in presence of DFMO or DFMO + putrescine for 3 days and serum starved for 24 h, and then cell extracts were prepared. After immunoprecipitation, JNK 1 activity was measured by phosphorylation of glutathione *S* transferase (GST) c-Jun (*A*) and protein level (*C*) was analyzed by Western blot analysis. Specific activity (*A*) is expressed as % control (means ± SD, $n = 3$) and was calculated from arbitrary units of activity and protein levels determined by densitometric scanning. *Significant difference compared with control using ANOVA ($P < 0.05$).

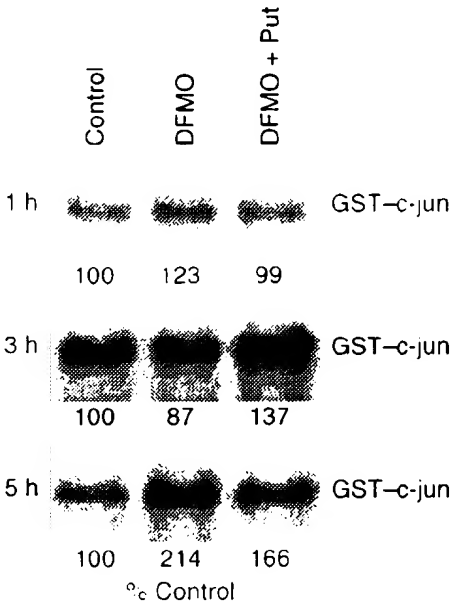

Fig. 8. Time course of effect of polyamine depletion on activity of JNK 1. IEC 6 cells were grown under control conditions for 3 days, serum starved for 24 h, and treated in presence and absence of DFMO or DFMO + putrescine for indicated times. JNK-1 activity was determined as described in MATERIALS AND METHODS.

and S phases within one cell cycle after plating Chinese hamster ovary (CHO) cells in the presence of DFMO. Coupled with our finding of no significant increase in apoptosis, these data indicate that polyamine depletion in IEC-6 cells caused cell cycle arrest.

Progression through the cell cycle is mediated by a phylogenetically conserved family of protein kinases known as CDKs. CDKs are composed of a catalytic subunit and a requisite positive regulatory subunit termed a cyclin (44). The CDK activities that govern cell cycle progression require coordination and regulation. In most cases, positive regulation is mediated at the level of cyclin accumulation (22, 25, 42). Negative regulation of CDK activity is achieved by phosphorylation of the catalytic subunit or via the binding of CDK inhibitory proteins that include $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ (41). Increased levels of CDK inhibitory proteins bind to cyclin-CDK complexes and render these complexes inactive. CDK inhibitory proteins have been implicated in negative regulation of the cell cycle by both internal and external signals (19, 54). Treatment of IEC-6 cells with DFMO increased the levels of $p27^{Kip1}$ and $p21^{Waf1/Cip1}$ (Fig. 4, *B* and *C*). The increase in $p21^{Waf1/Cip1}$ was significantly greater than the increase in $p27^{Kip1}$. Induction of p21 has been reported to be both p53 dependent and p53 independent based on the type of stimulus (34). Activation of p53 turns on the transcription of $p21^{Waf1/Cip1}$, which binds to and inhibits CDKs, causing the accumulation of the hypophosphorylated form of retinoblastoma protein. This form of retinoblastoma protein binds to E2F, which is a specific transcription factor for S phase gene expression, prevents the release of E2F, and blocks the G_1-S transition (12). Polyamine-depleted cells showed a significant induction of p53 protein (Fig. 4*A*), suggesting p53-dependent induction of $p21^{Waf1/Cip1}$.

The MAPK and SAPK pathways, which include the ERKs and JNK, respectively, link cell surface and nuclear events. Downstream effects of activated MAPK include phosphorylation of the substrates c-Fos and c-Jun, which in turn stimulates cell cycle progression. The current experiments clearly demonstrate that polyamine depletion decreased ERK-2 protein level while increasing ERK-2 activity. Addition of putrescine maintained the basal level of ERK-2 activity (Fig. 6). In contrast, ERK-1 protein was not affected by polyamine depletion, but ERK-1 activity also was increased (Fig. 5). This was a surprising and unanticipated correlation between increased MAPK activity and growth inhibition. These results are in contrast to the existing paradigm, which suggests concomitant increases in MAPK activity and cell proliferation. Pumiglia and Decker (45) first described a novel paradigm in which the MEK/MAPK pathway negatively regulates CDK activity and mediates cell cycle arrest. Soon thereafter, constitutively active MEK-1 was shown to induce epithelial differentiation and growth inhibition in Madin-Darby kidney cells (49) and MAPK was reported to mediate inhibition of proliferation in smooth muscle cells (3).

Because polyamine depletion inhibited cell growth and induced MAPK, we presumed that DFMO might cause stress and induce stress-regulated kinase (JNK) activity. DFMO treatment completely depleted putrescine within 3 h, whereas spermidine and spermine depletion was evident after 24 h and 3 days, respectively, in cultured intestinal epithelial cells (36). We observed a sustained increase in JNK activity when cells were grown in the presence of DFMO. Increased JNK activity was the earliest event observed (Fig. 8) and remained elevated up to 4 days (Fig. 7). Taken together, these results indicate a link between cell growth and activation of MAPK and SAPK. Recently, a role for oncogenic Ras and the MAPK pathway in p53 modulation and function has been revealed in both human and rodent cells. High expression of Ras or activation of the Mos/MAPK pathway induces wild-type p53 levels and causes a permanent growth arrest, similar to cellular senescence (16, 52). In a cell line defective in the MAPK pathway and in p53 expression, increased expression of ERK-2 restores the normal level of p53, clearly placing ERK-2 in a pathway that regulates the steady-state level of p53. MAPK has been shown to phosphorylate residue 73 or 83 of murine p53 in vitro (2, 38); p53 is involved in several different aspects of cell cycle arrest, apoptosis, control of genome integrity, and DNA repair (64, 65). Sustained increases in JNK also result in apoptosis in some cell lines by activating p53 and inducing CDK inhibitor proteins (8, 23, 32, 52, 64).

In summary, our results show that polyamine depletion of IEC-6 cells leads to cell cycle arrest in G_1 without causing apoptosis. Inhibition of growth was accompanied by increased levels of p21, p27, and p53. Taken together, these data suggest that in the absence of polyamines increased MAPK/JNK induces p53, which in turn may increase the transcription of p21, inhibiting CDK and blocking cell cycle progression. This is the first report that polyamine depletion activates signal transduction pathways leading to cell cycle arrest.

We thank Dr. Danis Buxton for the GST-c-Jun protein and Marion Merrell Dow (Cincinnati, OH) for providing DFMO. We are thankful to Mary Jane Viar and Pat Blanner for technical assistance and to Danny Morse for help with the figures.

This work was supported by National Institutes of Health Grants DK-16505 (to L. R. Johnson) and HL-48308 (to T. B. Patel).

Address for reprint requests: L. R. Johnson, Dept. of Physiology and Biophysics, University of Tennessee, Memphis, 894 Union Ave., Memphis, TN 38163.

Received 21 August 1998; accepted in final form 4 December 1998.

REFERENCES

1. **Adam, L. P., M. T. Franklin, G. J. Raff, D. R. Hathway.** Activation of mitogen-activated protein kinase in porcine carotid arteries. *Circ. Res.* 76: 183–190, 1995.
2. **Agrawal, M. L., W. R. Taylor, O. B. Chernon, and G. R. Strak.** The p53 network. *J. Biol. Chem.* 273: 1–4, 1998.
3. **Bornfeldt, K. E., J. S. Campbell, H. Koyama, G. M. Argast, C. L. Leslie, and E. W. Raines.** The mitogen-activated protein kinase pathway can mediate growth inhibition and proliferation in smooth muscle cells. Dependence on the availability of downstream targets. *J. Clin. Invest.* 100: 875–885, 1997.
4. **Bradford, M. M.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254, 1976.
5. **Charollis, R. H., and J. Mester.** Resumption of cell cycle in BALB/c-3T3 fibroblasts arrested by polyamine depletion: relation with "competence" gene expression. *J. Cell. Physiol.* 137: 559–564, 1988.
6. **Chellappan, S. P., S. Hiebert, M. Mudryj, J. M. Horowitz, and J. R. Nevins.** The E2F transcription factor is a cellular target for the RB protein. *Cell* 65: 1053–1061, 1991.
7. **Chen, P. L., P. Scully, J. Y. Shew, J. Y. Wang, and W. H. Lee.** Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell* 58: 1193–1198, 1989.
8. **Chen, Y.-R., X. Wang, D. Templeton, R. J. Davis, and T.-H. Tan.** The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation. *J. Biol. Chem.* 271: 31929–31936, 1996.
9. **Cowley, S., H. Paterson, P. Kemp, and C. J. Marshall.** Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell* 77: 841–852, 1994.
10. **Derijard, B., M. Hibi, I.-H. Wu, T. Barett, B. Su, T. Deng, M. Karin, and R. J. Davis.** JNK1: a protein kinase stimulated by UV light and Ha Ras that binds and phosphorylates the c-jun activation domain. *Cell* 76: 1025–1037, 1994.
11. **Dimri, G. P., M. Nakanishi, P. Y. Desprez, J. R. Smith, and J. Campisi.** Inhibition of E2F activity by the cyclin-dependent protein kinase inhibitor P21 in cells expressing or lacking a functional retinoblastoma protein. *Mol. Cell. Biol.* 16: 2987–2997, 1996.
12. **El-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein.** WAF1, a potential mediator of P53 tumor supression. *Cell* 75: 817–825, 1993.
13. **Fredlund, J. O., and M. Oredsson.** Normal G_1/S transition and prolonged S phase within one cell cycle after seeding cells in the presence of an ornithine decarboxylase inhibitor. *Cell Prolif.* 29: 457–466, 1996.
14. **Fredlund, J. O., and M. Oredsson.** Impairment of DNA replication within one cell cycle after seeding cells in the presence of polyamine-biosynthesis inhibitor. *Eur. J. Biochem.* 237: 539–544, 1996.
15. **Frey, M. R., M. L. Saxon, S. X. Zhao, A. Rollins, S. S. Evans, and J. D. Black.** Protein kinase C isozyme-mediated cell cycle arrest involves induction of $P21^{waf1/cip1}$ and $P27^{kipl}$ and hypophosphorylation of the retinoblastoma protein in intestinal epithelial cells. *J. Biol. Chem.* 272: 9424–9435, 1997.
16. **Fukasawa, K., and G. F. Vande Woude.** Synergy between the Mos/mitogen-activated protein kinase pathway and loss of P53 function in transformation and chromosome instability. *Mol. Cell. Biol.* 17: 506–518, 1997.
17. **Gupta, S., T. Barrett, A. J. Whitmarsh, J. Cavanagh, H. K. Sluss, B. D'erijard, and R. J. Davis.** Selective interaction of JNK protein kinase isoforms with transcription factors. *EMBO J.* 15: 2760–2770, 1996.
18. **Harada, J. J., and D. R. Morris.** Cell cycle parameters of Chinese hamster ovary cells during exponential, polyamine-limited growth. *Mol. Cell Biol.* 1: 594–599, 1981.
19. **Harper, J. W., and S. J. Elledge.** CDK inhibitors in development and cancer. *Curr. Opin. Genet. Dev.* 6: 56–64, 1996.
20. **Hengst, L., V. Dulic, J. M. Slingerland, E. Lees, and S. I. Reed.** A cell cycle regulated inhibitor of cyclin-dependent kinases. *Proc. Natl. Acad. Sci. USA* 9: 5291–5295, 1994.
21. **Hibi, M., A. Lin, T. Smeal, A. Minden, and M. Karin.** Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev.* 7: 2135–2148, 1993.
22. **Jeffrey, P. D., A. A. Russo, K. Polyak, E. Gibbs, J. Hurwitz, J. Massague, and N. P. Pavletich.** Mechanism of CDK activation revealed by the structure of cyclin A-CDK2 complex. *Nature* 376: 313–320, 1995.
23. **Johnson, N. L., A. M. Gardner, K. M. Diener, C. A. Lange-Carter, J. Gleavy, M. B. Jarper, A. Minden, M. Karin, L. I. Zon, and G. L. Johnson.** Signal transduction pathways regu-

lated by mitogen activated/extracellular response kinase kinase kinase induce cell death. *J. Biol. Chem.* 271: 3229–3237, 1996.
24. **Karin, M.** Regulation of AP1 activity by mitogen-activated protein kinases. *J. Biol. Chem.* 270: 16483–16186, 1995.
25. **Kiyokawa, H., R. D. Kineman, T. K. Manova, V. C. Soares, E. S. Hoffman, M. Ono, D. Khanam, A. C. Hayday, L. A. Frohman, and A. Koff.** Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function $P27^{kip1}$. *Cell* 85: 721–732, 1996.
26. **Klemke, R. L., S. Cai, A. L. Giannini, P. J. Gallagher, P. de Lanerolle, and D. A. Cheresh.** Regulation of cell motility by mitogen-activated protein kinase. *J. Cell Biol.* 137: 481–492, 1997.
27. **Ko, L. J., and C. Prives.** P53: puzzle and paradigm. *Genes Dev.* 10: 1054–1072, 1996.
28. **Kyriakis, J. M., P. Banerjee, E. Mikolakaki, T. Dai, E. A. Rubie, M. F. Ahmad, J. Avruch, and J. R. Woodgett.** The stress-activated protein kinase subfamily of c-jun kinases. *Nature* 369: 156–160, 1994.
29. **Lai, K., H. Wang, W. S. Lee, M. K. Jain, M. E. Lee, and E. Haber.** Mitogen activated protein kinase phosphatase-1 in rat arterial smooth muscle cell proliferation. *J. Clin. Invest.* 98: 1560–1567, 1996.
30. **Lange-Carter, C. A., and G. L. Johnson.** Ras dependent growth factor regulation of MEK kinase in PC12 cells. *Science* 265: 1458–1461, 1994.
31. **Levine, A. J.** P53, the cellular gatekeeper for growth and division. *Cell* 88: 323–331, 1997.
32. **Lowe, S. W., H. E. Ruley, T. Jacks, and D. E. Houseman.** P53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 74: 957–968, 1993.
33. **Lundberg, A. S., and R. A. Weinberg.** Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. *Mol. Cell. Biol.* 18: 753–761, 1998.
34. **MacLeod, K. F., N. Sherry, G. Hannon, D. Beach, T. Tokino, K. Kinzler, B. Vogelstein, and T. Jacks.** p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. *Genes Dev.* 9: 935–944, 1995.
35. **McCormack, S. A., P. M. Blanner, B. J. Zimmerman, R. Ray, H. M. Poppelton, T. B. Patel, and L. R. Johnson.** Polyamine deficiency alters EGF receptor distribution and signal effectiveness in IEC-6 cells. *Am. J. Physiol.* 274 (*Cell Physiol.* 43): C192–C205, 1998.
36. **McCormack, S. A., M. J. Viar, and L. R. Johnson.** Polyamines are necessary for cell migration by a small intestinal crypt cell line. *Am. J. Physiol.* 264 (*Gastrointest. Liver Physiol.* 27): G367–G374, 1993.
37. **McMohan, S. B., and J. G. Monroe.** Role of primary response genes in generating cellular response to growth factors. *FASEB J.* 6: 2707–2715, 1992.
38. **Milne, D. M., D. G. Campbell, F. B. Caudwell, and D. W. Meek.** Phosphorylation of the tumor suppressor protein P53 by mitogen activated protein kinase. *J. Biol. Chem.* 269: 9253–9260, 1994.
39. **Milne, D. M., L. E. Campbell, D. G. Campbell, and D. W. Meek.** P53 is phosphorylated in vitro and in vivo by an ultraviolet radiation-induced protein kinase characteristic of the c-jun kinase. *J. Biol. Chem.* 270: 5511–5518, 1995.
40. **Minden, A., A. Lin, T. Smeal, B. Derijard, M. Cobb, R. Davis, and M. Karin.** c-Jun N-terminal phosphorylation correlates with activation of the JNK subgroup but not the ERK subgroup of mitogen-activated protein kinases. *Mol. Cell. Biol.* 14: 6683–6688, 1994.
41. **Morgan, D. O.** Principles of CDK regulation. *Nature* 374: 131–134, 1995.
42. **Nigg, E. A.** Cyclin dependent protein kinase: key regulators of the eukaryotic cell cycle. *Bioessays* 17: 471–480, 1995.
43. **Pegg, A. E.** Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Res.* 48: 759–774, 1988.
44. **Pines, J.** Cyclins and cyclin dependent kinases: a biochemical view. *Biochem. J.* 308: 697–711, 1995.
45. **Pumiglia, K. M., and S. J. Decker.** Cell cycle arrest mediated by the MEK/mitogen-activated protein kinase pathway. *Proc. Natl. Acad. Sci. USA* 94: 448–452, 1997.
46. **Quaroni, A., J. Wands, R. L. Trelstad, and K. J. Isselbacher.** Epitheloid culture from rat small intestine. Characterization by morphologic and immunologic criteria. *J. Cell Biol.* 80: 218–265, 1979.
47. **Raingeaud, J., S. Gupta, J. S. Rogers, M. Dickens, J. Han, R. J. Ulevitch, and R. J. Davis.** Pro inflammatory cytokines and environmental stress cause P38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem.* 270: 7420–7426, 1995.
48. **Russell, D. H., and B. G. M. Durie.** Ornithine decarboxylase: a key enzyme in growth. *Proc. Cong. Res. Ther.* 8: 43–58, 1978.
49. **Schramek, H., E. Feifel, E. Healy, and V. Pollack.** Constitutively active mutant of mitogen-activated protein kinase kinase MEK1 induces epithelial differentiation and growth inhibition in Madin Darby canine kidney-C7 cells. *J. Biol. Chem.* 272: 11426–11433, 1997.
50. **Seger, R., D. Seger, A. A. Reszka, E. S. Munar, H. Eldar-Finkelman, G. Dobrowolska, A. M. Jensen, J. S. Campbell, E. H. Fisher, and E. G. Kreb.** Over-expression of mitogen-activated protein kinase kinase (MAPKK) and its mutant in NIH3T3 cells: evidence that MAPKK's involvement in cellular proliferation is regulated by phosphorylation of serine residue in its kinase subdomains VII and VIII. *J. Biol. Chem.* 269: 25699–25709, 1994.
51. **Seidenfeld, J., A. L. Block, K. A. Komar, and M. F. Naujokas.** Altered cell cycle phase distributions in cultured human carcinoma cells partially depleted of polyamines by treatment with difluoromethylornithine. *Cancer Res.* 46: 47–53, 1986.
52. **Serrano, M., A. W. Lin, M. E. McCurrach, D. Beach, and S. W. Lowe.** Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 88: 593–602, 1997.
53. **Servant, M. J., E. Giasson, and S. Meloche.** Inhibition of growth factor induced protein synthesis by selective MEK inhibitor in aortic smooth muscle cells. *J. Biol. Chem.* 271: 16047–16052, 1996.
54. **Sherr, C. J., and J. M. Roberts.** Inhibitors of mammalian G_1 cyclin-dependent kinases. *Genes Dev.* 9: 1149–1163, 1995.
55. **Silen, W.** Gastric mucosal defense and repair. In: *Physiology of the Gastrointestinal Tract*, edited by L. R. Johnson. New York: Raven, 1987, p. 1055–1069.
56. **Sluss, H. K., T. Barrett, B. Derijard, and R. J. Davis.** Signal transduction by tumor necrosis factor mediated by JNK protein kinase. *Mol. Cell. Biol.* 14: 8376–8384, 1994.
57. **Tabor, C. W., and H. Tabor.** Polyamines. *Annu. Rev. Biochem.* 53: 749–790, 1984.
58. **Traverse, S., N. Gomez, H. Paterson, C. Marshall, and P. Cohen.** Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effect of nerve growth factor and epidermal growth factor. *Biochem. J.* 288: 351–355, 1992.
59. **Van Dam, H., D. Wilhelm, I. Herr, A. Steffen, P. Herrlich, and P. Angel.** ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-jun induction in response to genotoxic agents. *EMBO J.* 14: 1798–1811, 1995.
60. **Wang, J. Y., and L. R. Johnson.** Role of ornithine decarboxylase in repair of gastric mucosal stress ulcers. *Am. J. Physiol.* 258 (*Gastrointest. Liver Physiol.* 21): G78–G85, 1990.
61. **Wang, J. Y., and L. R. Johnson.** Polyamines and ornithine decarboxylase during repair of duodenal mucosa after stress in rats. *Gastroenterology* 100: 333–343, 1991.
62. **Westwick, J. K., A. E. Bielawska, G. Dbaibo, Y. A. Hannun, D. T. Dudley, L. Pang, S. J. Decker, A. J. Bridges, and A. R. Saltiel.** A synthetic inhibitor of the mitogen activated protein kinase cascade. *Proc. Natl. Acad. Sci. USA* 92: 7686–7689, 1995.
63. **Whitmarsh, A. J., P. Shore, A. D. Sharrocks, and R. J. Davis.** Integration of MAP kinase signal transduction pathways at the serum response element. *Science* 269: 403–406, 1995.
64. **Yu, R., A. A. Shitl, T. H. Tan, I. B. Roninson, and A.-N. T. Kong.** Adriamycin activates c-jun N-terminal kinase in human leukemia cells: a relevance to apoptosis. *Cancer Lett.* 107: 73–81, 1996.